REMARKS

Reconsideration of this application is requested in view of the amendments to the claims and the remarks presented herein.

The claims in the application are claims 29 to 32, 35, 38 to 40, 42 to 44 and 47, all other claims having been cancelled.

Applicants are submitting herewith the sequence listing and the diskette as requested by the Examiner in order to insert the sequence identifying number in line 24 of page 15 and line 22 of page 28.

All of the claims were rejected under 35 USC 112, first paragraph, as containing subject matter not described in the specification so as to enable one reasonably skilled in the art to effect the invention. The Examiner states that the specification does not provide support for the structures as recited in the claims since they do not recite for the middle Lys residue as being 9 to 13 residues as defined by the variables n and m. The specification allegedly fails to provide a description of the structure wherein the Lys contains more than 3 residues as given in the figures.

Applicants respectfully traverse the Examiner's rejection since there is full

support in the specification for the four distinct structures of the claimed carbohydrate peptide conjugates. The support can be found in the passage corresponding to the box portion of page 7 filed herewith wherein it is specified that the claimed conjugate "comprises at least 3 lysines and up to 15 lysine residues covalently linked to one another". Then, from the whole structure is depicted in Figure 1, the only allowed variability exclusively concerns the central lysine residue. For the conjugates of Formula "B4-T4-M (first one)" and "B2-T2-M", the number of lysine residues is at least 3 and up to 15 when n is an integer from 1 to 13. Similarly, for the conjugates of Formula B8-T-8-M and B4-T 4-M (second one), the number of lysine residues is at least 3 and up to 15 when m is an integer from 1 to 9. Moreover, Example 1 discloses the complete protocol for the synthesis of a preferred embodiment of the claimed carbohydrate peptide conjugate. One skilled in the art starting from the exhaustive method of synthesis disclosed in Example 1, is fully capable of adapting the protocol for synthesizing any of the claimed conjugates. Therefore, there is full support for the residues and withdrawal of this rejection is requested.

The Examiner further rejected the claims on 35 USC 112, first paragraph, as alleging the specification fails to provide a written description for a vaccine or an immunogenic composition effective against tumors using the conjugate. The Examiner further alleges that the specification fails to provide an adequate description of the different derivatives of the carbohydrate tumor antigen and only provides a general description and its exemplification is nil or directed to the single sugar moiety galactosyl-N-acetyl serine.

Applicants respectfully traverse the Examiner's objection concerning the written description for a vaccine or an immunogenic composition since the experimental results shown in Examples 3 and 4 clearly demonstrate the usefulness of the claimed conjugates as the active ingredients of either an immunogenic composition or a vaccine composition. Example 4 clearly shows that a composition containing a carbohydrate peptide conjugate of the invention induce the production of antibodies which are recognized by a human adenocarcinoma. Such a composition clearly consists in an immunogenic composition since it is able to raise the production of specific antibodies against a tumor.

Moreover, the results in Example 3 show that a composition containing a carbohydrate peptide conjugate of the invention induces the protection of adenocarcinoma-bearing mice. These results clearly demonstrate that a composition of the invention may also consist of an effective vaccine composition to protect cancer bearing animals. Moreover, the successful results obtained with an immunogenic composition or a vaccine composition of the invention are directly transposable to humans. A support for said transposition to cancer bearing human is illustrated notably by the enclosed article of Longenecker et al (Ann. N.Y. Acad. Sci. Vol. 690, pp. 276-291). In this article. Longenecker et al shows that an antigenic construct containing a synthetic carbohydrate antigen induces, <u>both in mice and in humans</u>, specific antibodies recognizing the synthetic tumor antigen as well as an anti-cancer protective immune response (see Table 5 on page 288).

It is true that the rays of a protective anti-cancer immune response was highly unpredictable when using prior art antigenic constructs but once it is shown that a specific antigenic construct and specifically, the carbohydrate peptide conjugates of the present invention raise an anti-cancer protective response in animals specifically, in mice, the efficiency of the implementation of these conjugates for treating humans is directly transposable. Applicants will concede the Examiner is perfectly correct when he underlines that the claimed carbohydrate peptide conjugates will not be effective for treating all types of tumors. Indeed, the claimed carbohydrate peptide conjugates will be efficient exclusively for inducing a protective immune response in patients suffering from cancers wherein the tumor express a carbohydrate moiety against which the immune response is sought. Therefore, the specification is enabling for the vaccines and immunogenic compositions.

With respect to the Examiner's objection to the specification for failing to provide an adequate description of the different derivatives of the carbohydrate tumor antigen, Applicants respectfully disagree with the same. A derivative in the general understanding of one skilled in the art consists of a compound than can be imagined to arise from a parent compound by replacement of one atom with another atom or a group of atoms. This is used extensively in organic chemistry to assist in identifying

compounds. The expression "derivative thereof" when this expression relates to a carbohydrate may, for example, be referred to in the definition included in the USPTO official classification, class 536, subclass 1.11, a copy of which is enclosed herewith. It is clear from the specification that every carbohydrate which consists of a natural tumor antigen and every carbohydrate moiety having chemical differences regarding the natural tumor antigen, but which may induce a protective immune response against the natural antigen can be used. It should be noted that the essential feature of the claimed carbohydrate peptide conjugates consist of the combination of 1) the specific structures of the dendrimeric polylysine residues or the lysine core, combined with 2) the presence of a B-cell epitope consisting of a carbohydrate moiety which, when associated with a T-cell epitope within the same antigenic construct, possess the property of inducing a protective anti-cancer immune response. Therefore, the specification is deemed to be enabling and withdrawal of these grounds of rejection is requested.

Claims 29 to 32, 35, 38 to 40, 42 to 44, 46 and 47 were rejected under 35 USC 112, second paragraph, as being indefinite. The Examiner deemed that claim 29 was unclear as to the attachment of the T and B to the Lys residue. Claim 31 was objected to in the recitation "at least 4 T-cell epitopes" as being confusing.

Applicants respectfully traverse these grounds of rejection since claims 29 and 31 are believed to be proper. The content of the specification makes clear and obvious to

one skilled in the art that the B and T epitopes attachment may be performed either through the α or the ε-amino group. The way according to which the core of the lysine residues is synthesized clearly sustains the availability of the attachment alternative. On pages 15 and 16 of the application as filed under "Reference compounds", from line 28 of page 15 to line 1 of page 16, there are discussed the synthesis. It is obvious to one skilled in the art that the step of removing the Fmoc protective groups render both the α and the ε-amino groups available for further chemical reactions including reactions for binding the B and the T epitopes to the lysine core. With respect to claim 31, the Examiner was correct since claimed conjugates contain in fact at least two T-cell epitopes and claim 31 has been amended accordingly.

The Examiner further objected to claim 29 in the expression "enabling multiple epitopes" as introducing uncertainty as to the conjugation or number of multiple epitopes. It was not clear to the Examiner whether several or multiple epitopes correspond exclusively to those present in the formula or not. The Examiner noted a misspelling as well. In claim 35, the Examiner objected to the expression "the carbohydrate is grafted". In claim 38, the Examiner was of the opinion that "T* antigen" was unclear since, in the Examiner's opinion, the specification did not provide positive support therefor. Claim 40 as objected to as being indefinite as to the other components of the vaccine and claim 46 was objected to as being indefinite in the induction of an immune response to only B-cells. Claim 47 was deemed to be a duplicate of claim 44.

Applicants respectfully traverse these grounds of rejection since the amended claims are believed to comply with the second paragraph of 35 USC 112. With respect to claim 35, a method of grafting is disclosed on page 17 enclosed herewith. For the conjugate named "B4-T4-M" wherein it is specified that "the synthesis of B4-T4-M was achieved by ultimately coupling 2 to T4-M (0.25 g of resin, 0.03 mmol) which was obtained as described above. The preparation of compound 2 is disclosed from line 20, of page 14 of the specification. The step of grafting the carbohydrate moiety involves the formation of a peptide bound, as is easily understood by one skilled in the art.

With respect to claim 38, the expression "T* antigen" is clearly supported on page 1 of the application, a copy of which is enclosed herewith and specifically, in line 22, wherein T* is defined as well as sialosyl-Tn. With respect to claim 40, the essential feature of the claimed vaccine consists of the conjugate which is the active ingredient to raise a protective anti-cancer immune response in the patient to which the vaccine is administered. The said vaccine beyond the carbohydrate peptide conjugate of the invention may also comprise an adjuvant of immunity or other conventional excipients which are to be included in normal vaccine compositions. With respct to claims 42 and 43, the objected to expression has been deleted and claim 46 has been cancelled.

With respect to the alleged redundancy of claims 44 and 47, it should be noted that claim 44 relates to a method for inducing an immune response and therefore, relates to the use of an immunogenic composition as claimed in claims 42 and 43. In contrast, claim 47 relates to a method of vaccination using a vaccine composition as claimed in claim 40. These are two distinct embodiments of the invention. Claim 44 is directed to the results shown in Example 4 wherein it is demonstrated an induction of anti-tumor antigens specific antibodies with a composition containing a carbohydrate peptide conjugate of the invention. Claim 47 is directed to the embodiment of Example 3 wherein the induction of an anti-tumor protective immune response with a carbohydrate peptide conjugate of the invention is shown. Therefore, the claims are patentably distinct.

All of the claims were rejected under 35 USC 103 as being obvious over the Chong et al patent for reasons of record. The Examiner states that Chong et al discloses in column 17, that the synthetic glycol conjugate may be used to produce vaccines eliciting antibodies against proteins or oligosaccharide and the suggested teachings of Chong et al would lead one skilled in the art to the claimed anti-tumor vaccine. The Examiner cites the Jondal patent as disclosing a conjugate of protein and tumor or bacterial antigen effective as anti-tumor or anti-bacterial antigens. The important aspect of the conjugation of protein-polysaccharide conjugates according to the Examiner is the desire to elicit both T and B cell response.

Applicants respectfully traverse these grounds of rejection since it is deemed that the combination of the prior art that the Examiner has made with the benefit of Applicants' teaching would not suggest Applicants' invention to one skilled in the art. It must be emphasized that Chong et al exclusively discloses dendrimeric conjugates which combine only <u>peptide</u> T and B-epitopes and Chong et al does not disclose or suggest any synthetic conjugate wherein the B-epitope is included in a carbohydrate moiety as detailed hereunder. In lines 39 to 48 of column 2, it is clear that the technical solution found by Chong et al to induce an immune response consists of using "synthetic peptides containing immune-dominant epitopes from Hi OMPS as additional antigens". The synthetic carbohydrate PRP oligomers that may be present in the conjugate disclosed by Chong et al are exclusively used as T-cell epitopes as can be seen from lines 32 to 37 of column 3.

From line 65 of column 3 through line 67 of column 5, Chong et al exclusively discloses synthetic conjugates wherein the B-cell epitope is only a peptide compound and more particularly, one of the P1, P2 and P6 protein from Haemophilus influenaz (Hib). It is clear in various portions of this portion of Chong et al, particularly line 62 of column 4, wherein the B-cell epitope of the P1 protein is cited, in line 8 of column 5 wherein the B-cell epitope of P2 is cited and in line 19 of column 5 wherein the B-cell epitope of P6 peptide is cited.

Similarly, the T-cell epitopes included in the synthetic conjugates of Chong et al consist also of peptides as it flows from several assays of Chong et al. In line 66 of column 3 through line 3 of column 4, ("the present invention relates to the provision of immunogenes and candidate vaccines made of peptides containing the amino acid sequences of various antigenic determinants (T-helper cell and B-cell epitopes) of the outer membrane proteins (P1, P2 and P6) of Hib)." It is also referred to the immunodominant T-cell epitope of P1 protein in lines 29 to 31 of column 5, the P2 protein in lines 40 to 42 of column 5 and P6 protein in lines 51 to 53 of column 5. The immunodominant T-cell epitope of at least one protein of Haemophilus influenza is cited in lines 62 to 66 to column 5. In line 47 of column 8 through line 3 of column 9, a specific embodiment of synthetic conjugate vaccine is provided wherein the synthetic PRP-carrier conjugate vaccine contained one peptide T-cell epitope as well as a peptide B-cell epitope. Moreover, from line 21 of column 10 through line 13 of column 11, Chong et al discloses methods for selecting exclusively peptide T-cell and B-cell epitopes.

It flows from the entire Chong et al disclosure that the synthetic conjugate disclosed contains exclusively <u>peptide</u> B and T-epitopes and that the PRP carbohydrate moiety is used as a carrier for these epitopes. As expressed by the term "PRP-carrier conjugate vaccine" which are found in lines 50 to 51 of column 6, it appears clear that in most of the embodiments of the synthetic conjugates of Chong et al, the B-epitope and

the T-epitope are born by the same peptide structure since the P1, P2 and P6 proteins contain both types of epitopes as can be seen from line 59 of column 4 through line 27 of column 5 for the B-epitopes in the P1, P2 and P6 proteins and from lines 29 to 61 of column 5 for the T-cell epitopes included in the P1, P2 and P6 proteins.

From the preceding analysis of the Chong et al disclosure, it is clear that when Chong et al suggests the usefulness of the synthetic conjugate to induce immunity towards tumor cells or to produce anti-tumor antibodies, Chong et al only suggests the use of peptide antigen for inducing an anti-tumoral immune response. One skilled in the art would find no technical guidance from Chong et al to have motivated him to manufacture a carbohydrate peptide conjugate such as the claimed conjugates and specifically, carbohydrate peptide conjugates wherein the tumor antigen which is used consists of a carbohydrate moiety containing a B-cell epitope and wherein the carbohydrate moiety is a tumor antigen.

Applicants again incorporate the prior remarks concerning the Schreiber reference, the Pardoll and the Dalgleish references cited in the prior response which has never been commented on by the Examiner. Applicants incorporate herewith the remarks in the June 5, 2001 response to not unduly burden the record. Particularly, the unpredicatability of obtaining a therapeutically efficient antibody response against tumor antigens using prior art vaccines is disclosed in the last paragraph on page 1620 and the

right-hand column of page 1621 of the Dalgleish reference. Also, the immune tolerance related to tumor antigens is clearly disclosed by Pardoll on page S44 the right-hand column and by Schrieber on pages 1256 and 12157. It flows from this analysis that one skilled in the art who had knowledge that raising antibodies against tumor antigens was a highly difficult test, would not have been motivated to replace the peptide B-epitope of the antigenic constructs of Chong et al by a carbohydrate moiety B-epitope.

The secondary Jondal patent does not overcome the deficiencies of Chong et al since Jondal discloses an antigenic construct consisting of a carrier molecule, namley, KLH which is fused to an antigenic moiety consisting of a peptide bearing T-cell epitope capable of binding a MHC Class I molecule and a carbohydrate component having the same immunogenic characteristics of the carbohydrate structure on the tumor cell infectious agent or the infected cells as can be seen from the Abstract. In the fourth paragraph of column 4 of Jondal, the antigenic constructs are aimed to induce a cytotoxic T-cell response against the carbohydrate structures and <u>never</u> an antibody response. Jondal exclusively discloses technical means, namely, antigenic peptides, that consists of peptides that bind the Class I MHC molecules and which induce a cytotoxic T-cell response as indicated in the third paragraph of column 5. The criteria for selecting such appropriate antigenic peptides are disclosed in the last paragraph of column 5 and the first paragraph of column 6 and is illustrated by the examples wherein <u>only</u> CTL responses are shown. One skilled in the art would have no motivation to combine the two references

which pertain to the induction of cytotoxic T-cell response of Jondal with Chong et al which pertains to antibody responses. There is no indication in Jondal that the constructs disclosed therein would be able to raise any antibody response against a tumor antigen. Therefore, the combination of the prior art does not teach Applicants' invention and withdrawal of this ground of rejection is requested.

With respect to the obviousness double patenting rejection, Applicants are submitting herewith a terminal disclaimer and the fee to obviate this rejection.

In view of the amendments to the claims and the above remarks, it is believed that the claims clearly point out Applicants' patentable contribution and favorable reconsideration of the application is requested.

Respectfully submitted,
Muserlian, Lucas and Mercanti

Charles A. Muserlian, 19,683
Attorney for Applicants
Tel. # (212) 661-8000

CAM:ds
Enclosures

US 3522 134 I

TECHNICAL BACKGROUND

1. Field of the invention

The present invention is directed to the field of immunotherapy and more particularly to a glycoconjugate, a composition and vaccine comprising the same and to the use thereof for enhancing the immune response and notably in cancer therapy and in therapeutic of infection caused by pathogenic agent against whom a humoral or a cellular immune response is necessary. The invention relates also to a diagnosis kit and a method for diagnosis of cancer.

2. Prior Art / Relevant Literature

As a result of aberrant glycosylation, cancer-associated carbohydrate antigens are exposed at the surface of tumor cells whereas they are hidden in normal cells (Ref. 1). Recent advances in immunology and in the identification of tumor specific antigens have renewed the interest for the development of cancer vaccines, and these exposed glycosidic B-cell epitopes have been considered as attractive targets for immunotherapy named "Active Specific Immunotherapy" (ASI) by Longenecker (Ref. 2). This approach involves immunization with a defined antigen to elicit a specific immune response to that antigen and could represent an alternative to the conventional cancer therapies.

Among the large number of known tumor markers, the Tn (a-GalNAc-Ser/Thr), the T* (b-Gal-(1→3)-a-GalNAc-Ser/Thr) and the sialosyl-Tn (a-NeuAc-(2→6)-a-GalNAc-Ser/Thr) antigens have been extensively studied since they are expressed on mucin-type glycoproteins by the majority of adenocarcinomas (Ref. 3). Indeed, several studies have shown some protection against tumors after immunization with these glycosidic antigens, in experimental or clinical studies. Using desialylated red blood cells, which are rich in T and Tn determinants, Springer observed a long-term effective protection against recurrence of human breast carcinoma (Ref. 3c, Ref. 4). An other group investigated the potential of ASI with desialylated ovine submaxillary mucin (d-OSM), which contains high density of the Tn epitope ; their

Several different T epitopes means between two and eight of T epitopes from different origins.

Several different B-epitopes means between two and eight of B-epitopes from different origins.

The poly-Lysine core of the present conjugate is called a dendrimer because it may be represented (Figure 1) as a star with multiple branches all substantially identical.

As stated earlier, multiple antigen peptide system have been described in 1988 by Tam (Ref. 13) that are based also on certain dendrimeric structure in which peptidic antigen are covalently conjugated to the branches of the latter.

Examples of suitable carriers comprise those having a structure based on a poly-Lysine core forming a multiple branches star, such as, for example a 8 or 4 branches star.

Thus the present invention in one of its preferred embodiment is directed to a conjugate comprising a dendrimeric structure based on a poly-Lysine core forming a 4 branches star, with an epitope T covalently bound to each of the branches and associated to a carbohydrate moiety (provided it is not a sialoside radical) containing an epitope B.

According to a further preferred embodiment of the present invention the multiple antigen glycopeptide (MAG) forming the conjugate according to the present invention comprises at least 3 lysines and up to 15 lysines residues covalently linked to one another. Most preferably the present conjugate comprises 3 lysines.

In a preferred embodiment, to the NH2 end of each lysine residue is bound at least one peptide comprising one epitope T bound to a lysine and at least a carbohydrate residue, being not a sialoside, optionally substituted, covalently bound to the end of said peptide opposite to the lysine and forming a B epitope.

In another preferred embodiment, to the NH_2 end of each lysine residue is bound at least one carbohydrate residue, being not a sialoside, optionnally substituted and forming a B-epitope bound to a lysine and at least a peptide

6852.08 (Calcd. 6853.35). Amino acid analysis : Ala 4 (4), Asp 4.4 (4), Ile 4 (4), Leu 4.1 (4), Lys 15.8 (15), Phe 4 (4), Thr 8.2 (8), Tyr 4.3 (4), Val 3.8 (4).

Multiple Antigen Glycopeptide B4-T4-M **4** :

The synthesis of B4-T4-M was achieved by ultimately coupling **2** to T4-M (0.25 g resin, 0.03 mmol) which was obtained as described above. Cleavage of the glycopeptide was accomplished with TFA/water/ethanedithiol (95/2.5/2.5, 30 ml). After purification by HPLC (gradient from 10% to 65%, 11.9 min retention time), the target glycopeptide was obtained (25 mg). ES MS : 8014.09 (Calcd. 8014.45). Amino acid analysis : Ala 4 (4), Asp 4.78 (4), Ile 4.09 (4), Leu 4.15 (4), Lys 16.31 (15), Phe 4 (4), Ser 3.81 (4), Thr 8.58 (8), Tyr 4.5 (4), Val 3.63 (4).

EXAMPLE 2

Immunological results : Antigenicity and Immunogenicity of T,$CD4^+$ epitope and of Tn antigen within the glycoconjugate MAG according to the invention

Materials and methods :

Mice

Six to eight week-old female inbred mice were used in all experiments. BALB/c mice were from Iffa Credo (L'Abresle, France).

Antigen presentation assay :

For the dose response assays, 10^5 T cell hybridomas 45G10 (specific for 103-115 poliovirus peptide) per well were cultured with 10^5 A20 cells (ATCC, TIB-208 Rockville, MD) with different antigen doses for 24 h in RPMI 1640 medium supplemented with 10 % Fetal calf serum, antibiotics 2 mM L-glutamine, 5 x 10^{-5} M 2-mercaptoethanol. After 24 h, supernatants were frozen for at least 2h at -70 °C. 10^4 cells / well of the IL-2 dependent CTLL cell line was cultured with 100 µl aliquot supernatant in 0,2 ml final volume. Two days later, [^{3}H] thymidine (0,3 µCi/well ; AS = 1Ci/mmol) was added and the cells were harvested 18 h later with an automated cell harvester. Incorporated thymidine was detected by scintillation counting.

T-cell proliferation assay :

Mice were immunized subcutaneously with 10 µg of T, B-T, T4-MAP, B4-MAP or B4-T4-MAP compounds emulsified in complete Freund's adjuvant. Ten days

Immune Responses of Mice and Human Breast Cancer Patients following Immunization with Synthetic Sialyl-Tn Conjugated to KLH Plus Detox Adjuvant

B. MICHAEL LONGENECKER,[a,b,d] MARK REDDISH,[a] RAO KOGANTY,[a] AND GRANT D. MACLEAN[c,d]

[a]*Immunotherapeutics Division Biomira Inc. Edmonton, Alberta, Canada*

[b]*Department of Immunology Faculty of Medicine University of Alberta Edmonton, Alberta, Canada*

[c]*Department of Medicine Faculty of Medicine University of Alberta Edmonton, Alberta, Canada*

[d]*Cross Cancer Institute Edmonton, Alberta, Canada*

Most human carcinoma cells display cell surface mucin molecules that differ from mucins expressed on normal epithelial cells.[1] Carcinoma-associated mucins often have shortened carbohydrate side-chains exposing normally cryptic O-linked core carbohydrate determinants such as Tn, sialyl-Tn, and the Thomsen-Friedenreich (TF) determinant.[2] Tn is composed of the first core sugar αGalNAc O-linked to serine or threonine in the core peptide of the mucin molecule (Tn, αGalNAc-O-Ser/Thr). Tn is the precursor to TF, which is generated by the addition of β galactose by a galactosyltransferase[3] (TF, Galβ1-3αGalNAc-O-Ser/Thr). As human breast carcinoma cells may be deficient in a key galactosyltransferase,[3] Tn is exposed, and premature sialation of Tn can occur leading to the formation of sialyl-Tn[4-6] (STn, DAcNeu2-6αGalNAc-O-Ser/Thr). All three epitopes, Tn, TF, and STn, are strongly expressed on human carcinoma cells[2,4,5] and may be associated with cancer progression and metastasis of human carcinomas.[2,7-9] Of particular note is the recently observed association between STn expression and poor prognosis.[5,7]

We have shown that active immunization with synthetic carbohydrate TF hapten conjugated to keyhole limpet hemocyanin (TF-KLH) plus RIBI adjuvant, following low-dose cyclophosphamide to inhibit suppressor cells, can inhibit tumor growth and prolong survival of mice bearing tumors expressing TF carbohydrate epitopes.[10] These encouraging animal model studies prompted us to conduct phase I clinical trials in patients with ovarian carcinoma with extensive metastatic disease.[11] These studies were designed to test the effects of subcutaneous injections

of TFα-KLH plus DETOX™ adjuvant in patients with ovarian cancer, following a single intravenous injection with low-dose cyclophosphamide. Most patients demonstrated a "classical" hapten-specific antibody response with an increase in IgM titer followed by an increase in IgG and IgA anti-TF antibodies.[11] The specificity of the humoral antibody response to the TF hapten was demonstrated by antibody binding on solid-phase synthetic and natural TF antigens, by complement-mediated cytotoxicity assays, and by specific hapten-inhibition. These results demonstrated that KLH is an acceptable carrier for the synthetic TF hapten in humans and that DETOX is an appropriate nontoxic adjuvant for the generation of high-titer specific anticarbohydrate responses in human cancer patients. We recently reported similar results in patients with breast carcinoma immunized with STn-KLH plus DETOX adjuvant.[12] In addition, some of these patients showed a clinical response or prolonged disease stability.[12] In the present communication, we examine the immune response to sialyl-TN (STn). STn was chosen as a target structure because its expression on mucins is a strong independent predictor of poor prognosis,[5,7] suggesting that it may have functional significance in the metastatic process. Our working hypothesis is that an antibody response to STn expressed on carcinoma cells might limit or block metastasis[13] and may also possibly trigger antibody-mediated cytotoxicity and inflammatory reactions.

MATERIALS AND METHODS

Antigens and Haptens

All haptens and immunoconjugates were provided by Biomira, Inc. (Edmonton, Alberta, Canada): αDGalNAc-OR (Tn); βDGal(1–3)αDLGalNAc-OR (TFα); αD-NeuNAc(2–6)αDGalNAc-OR (sialyl-Tn); and αDNeuNAc(2–6)βDGalNAc-OR (βsialyl-Tn). R is the crotyl linker arm that can be used for covalent attachment of the hapten to a protein carrier. Sialyl-Tn-Serine hapten (αDNeuNAc(2–6)αD-GalNAc-O-Serine) was also synthesized.

Active Specific Immunotherapy Formulation Preparation for Human Use

Sialyl-Tn-KLH was provided as a sterile, pyrogen-free, pharmaceutically acceptable formulation by Biomira, Inc. DETOX (Ribi ImmunoChem Research, Inc., Hamilton, Montana) is a sterile, pyrogen-free preparation[14] and is formulated as a lyophilized oil droplet emulsion containing monophosphoryl lipid A and cell wall skeleton from *Mycobacterium phlei.* Immediately before injection, the STn-KLH was reconstituted with phosphate-buffered saline solution (PBS) and added to the lyophilized DETOX to give a final volume of approximately 1.0 ml at a final concentration of STn-KLH of 100 μg. The final mixture was administered as one half volume (~0.5 ml) of the 100 μg dose injected subcutaneously into each of two sites, alternating each treatment between upper arms (deltoid region) and anterolateral thighs.

Mouse Immunizations

Mice were immunized (10 per group) subcutaneously on days 0, 14, and 28 with STn-KLH emulsified in Ribi Adjuvant System (RAS). Doses ranging between

0.25 and 100 μg all delivered a total volume of 0.2 ml split into two injection sites. Immune sera were obtained on days 12, 26, and 40.

ELISA for Anti-STn Antibodies

Microtiter 96-well plates were coated with ovine submaxillary mucin[15,16] or with hapten-HSA conjugates. Control wells were coated with HSA only. Coated plates were blocked with 0.8% gelatin. Serial dilutions of sera were incubated on the antigen-coated plates at room temperature for 1 hour, after which the wells were thoroughly washed. Alkaline phosphatase-labeled specific anti-mouse or anti-human IgG or IgM (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland) antibodies were added to appropriate wells and incubated at room temperature for 1 hour. Each plate was then thoroughly washed and *p*-nitrophenyl phosphate substrate was added to each well. After 30 minutes at room temperature, 1 *M* HCl was added to each well to stop the enzyme reaction, and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader. Positive control high-titer patient sera were used on each plate to ensure reproducibility of results among plates and assays. Background optical density readings on HSA-coated wells were subtracted from readings obtained on STn-HSA–coated wells. The results of the titration are reported as the reciprocal of the highest serum dilution at which the optical density was greater than 0.12.[11]

Hapten Inhibition of the Anti-STn ELISA

An appropriate dilution of immune serum calculated to give an optical density of approximately 1.0 when tested with an ELISA with STn-HSA or ovine submaxillary mucin on the solid phase was mixed with the appropriate dilution of hapten in microtiter plates. All dilutions were made in phosphate-buffered saline solution (PBS), pH 7.4. The hapten-serum mixtures were incubated overnight at 2–6°C and the next morning transferred to ELISA plates containing solid-phase STn-HSA or ovine submaxillary mucin. The covered ELISA plate was then incubated at room temperature for 1 hour, and the ELISA was developed as just described. Other hapten inhibition studies were similarly carried out except that a kinetic read method was performed using the V_{max} plate reader (Molecular Devices). A total of 30 readings were taken and a rate kinetic determined by regression expressed as milli optical density per minute.

Complement-Mediated Lysis

DU4475 cells[17] were labeled with ^{51}Cr as previously described.[20] Fifty microliters of diluted sera were mixed with 50 μL of 10^4 labeled DU4475 target cells. Plates were incubated at 4°C for 1 hour and then washed using 100 μL of PBS and 1% bovine serum albumin. One hundred microliters of 10% complement in PBS (Lo Tox H Rabbit Complement, Cedarlane Labs. Ltd., Hornby, ON, Canada) were added to all wells and incubated for 1 hour at 37°C. The reaction was stopped by adding 75 μL of cold PBS, and the plates were centrifuged at 1,000 rpm for 10 minutes. A fraction of the supernatant was collected and counted in a gamma counter. Control wells containing target cells in PBS alone, complement alone, and Triton-10X (total release) were included in each plate. The percentage of specific ^{51}Cr released was calculated as previously described.[12]

Delayed Type Hypersensitivity Assay

The delayed type hypersensitivity (DTH) footpad assay was conducted in CAF_1 mice as previously described.[18] Hapten-specific DTH was tested with STn-HSA.

Clinical Trial Design

Seven patients were immunized with 100 μg of STn-KLH plus DETOX as previously described,[12] and these patients had histologically proven breast cancer with clinical or radiologic evidence of metastatic disease. The first six patients were part of a phase I study previously described[12] and the seventh patient was enrolled in an ongoing phase II study. A further six patients who received 25 μg of STn-KLH plus DETOX[12] were evaluated for their clinical response. All patients gave valid written informed consent and were aware of the phase I nature of this program.

Three days before the first active specific immunotherapy (ASI), each patient was treated with a single intravenous bolus treatment of cyclophosphamide, 300 mg/m^2 (with an antiemetic and 1,000 ml of normal saline solution intravenously for hydration).

For each patient, the first four ASI treatments were scheduled at 2 weekly intervals. Eligibility for a further four ASI treatments depended on subsequent evaluation, including the absence of significant toxicity, evidence of disease stability or even clinical response, and evidence of an immune response to the STn epitope. The four "booster" immunizations were to be given at 4 weekly intervals.

RESULTS

Antibody Response of CAF_1 Mice after Immunization with STn-KLH Plus DETOX Adjuvant

Six groups of 10 mice each were immunized three times with 0.25, 0.50, 1.0, 10, 25, and 100 μg of STn-KLH, respectively. Serum samples were collected 12 days after each immunization, and the specific IgM and IgG titers of each mouse were determined by kinetic ELISA on both ovine submaxillary mucin (OSM) and synthetic STn-HSA solid phases. Ovine submaxillary mucin was chosen because it is a convenient natural source of repeating sialyl-Tn determinants, which are O-linked on the mucin molecule.[15,16]

Preimmune sera showed no significant anti-STn-HSA or OSM binding. All mice in all dosage groups developed maximal IgG titers after the third immunization. The titers were comparable when tested on either STn-HSA or OSM (data not shown). FIGURES 1 and 2 show the development of IgG titers after the second and third immunization, demonstrating that all 10 mice immunized with synthetic STn conjugated to KLH developed high titers against OSM even at the lowest (0.25 μg) dose.

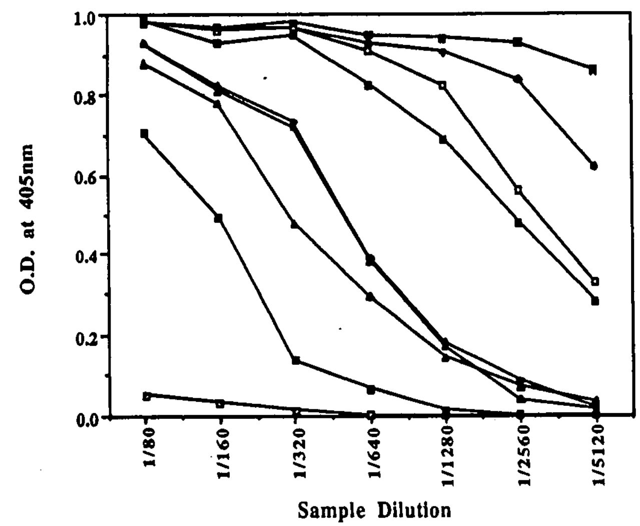


FIGURE 1. Murine polyclonal anti-STn antibodies were titrated on an ovine submaxillary mucin solid phase. CAF_1 mice (n = 10) were immunized subcutaneously with 0.25 μg of STn-KLH on days 0 and 14. The immune serums tested here were obtained on day 26.

Specificity of Murine Anti-STn-HSA and Anti-OSM Polyclonal Antibodies after Immunization with STn-KLH Plus DETOX

The sera generated in the experiments just described were tested with various synthetic haptens for inhibition of binding to STn-HSA or OSM. α2→6 STn-crotyl is the hapten that was used for conjugation to KLH by way of the crotyl linker arm to generate the STn-KLH immunogen used in all the studies reported in this paper. In FIGURE 3, a comparison is made between the structures of α2→6 STn-crotyl; β2→6 STn-serine; and α2→6 STn-serine. The STn-O-serine is the "natural" hapten as STn is naturally O-linked on mucins through serine. β2→6 STn-O-serine is the unnatural β anomer of sialyl-Tn. FIGURE 4 demonstrates that both STn-crotyl and STn-O-serine (alpha) produce essentially equivalent and complete inhibition of binding of the anti-STn-HSA (FIG. 4A) and anti-OSM (FIG. 4B) IgG antibodies.

Specificity of Murine Anti-OSM IgG Monoclonal Antibodies Induced after Immunization with STn-KLH Plus DETOX

To ascertian the specificity on a clonal level, we generated anti-OSM IgG producing B-cell hybridomas from the spleen cells of mice immunized with STn-KLH plus DETOX. Several clones were analyzed with virtually identical results. Hapten inhibition studies demonstrated that only synthetic STn (either STn-crotyl or STn-serine), but not related synthetic structures such as TF, Tn, and lactose haptens, inhibited specific anti-OSM monoclonal antibody (MAb) binding (TABLE 1). In further hapten inhibition studies, we compared the three different forms of synthetic sialyl-Tn (FIG. 3) for hapten inhibition. The pattern of inhibition of the

MAbs was virtually identical to that of the polyclonal antibodies already described with both STn-crotyl and STn-O-serine producing equivalent and complete inhibition of binding of the MAbs, whereas the unnatural β anomer of STn did not produce any significant inhibition of binding (FIG. 5).

Anti-Tumor Cell Reactivity of Monoclonal Antibodies Generated after Immunization with STn-KLH

Three MAbs were tested for binding to the Daudi human lymphoma cell line, which strongly expresses STn epitopes. FIGURE 6 shows the binding of B72.3, a well-known anti-STn MAb,[1] and MAb B195.2H5 generated from STn-KLH immunized mice to Daudi tumor cells. TABLE 2 demonstrates that the tumor cell binding of B72.3 and our new B195.2H5 anti-STn MAb generated following immunization with STn-KLH is inhibited by OSM in a dose-dependent fashion, confirming the anti-STn reactivity of the MAbs.

Anti-STn DTH Reactions Stimulated after Immunization of Mice with STn-KLH

FIGURE 7 demonstrates that small doses (1.0 μg per subcutaneous immunization) but not larger doses (10 and 25 μg per immunization) stimulated significant DTH reactions that peak at 48 hours after footpad challenge. FIGURE 7 also demonstrates an inverse relation between the antibody response and DTH as a function of antigen dose.

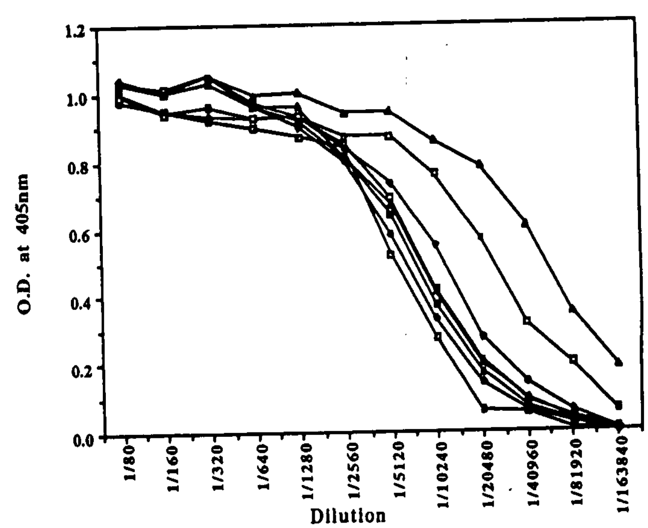
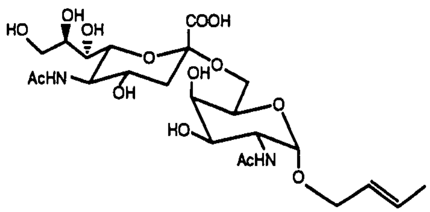
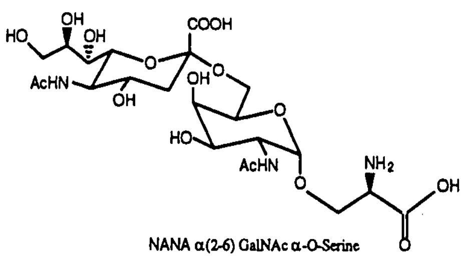


FIGURE 2. Murine polyclonal anti-STn antibodies were titrated on ovine submaxillary mucin solid phase. CAF_1 mice (n = 10) were immunized subcutaneously with 0.25 μg of STn-KLH on days 0, 14, and 28. The immune serums tested here were obtained on day 40.



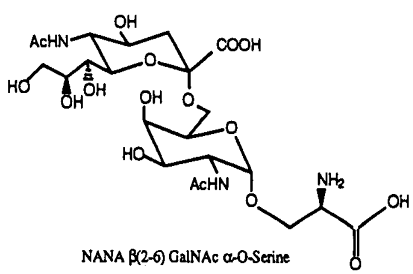
NANA α(2-6) GalNAc α-O-Crotyl

(ST_N- CROTYL)



NANA α(2-6) GalNAc α-O-Serine

(ST_N- O - SERINE)



NANA β(2-6) GalNAc α-O-Serine

(β(2-6) ST_N - O - SERINE)

FIGURE 3. Structures of the STn-related haptens are shown schematically.

Immunization of Breast Cancer Patients Using STn-KLH Plus DETOX

One hundred micrograms of STn-KLH were used with DETOX adjuvant to immunize patients with metastatic breast cancer. STn-HSA in a solid phase ELISA showed that all six patients developed IgM and IgG specific for the synthetic STn hapten. We also detected IgG antibodies reactive with synthetic STn-HSA as well

as natural STn determinants expressed on OSM (TABLE 3). In all patients, IgG antibodies to STn-HSA developed more rapidly and reached higher titers than did those detected on OSM (TABLE 3). Nevertheless, significant IgG binding to OSM developed in all patients with maximum titers ranging from 1 : 80–1 : 640.

After immunization, most patients were shown to develop increased titers of complement-mediated cytotoxic antibodies, partially inhibited by synthetic STn

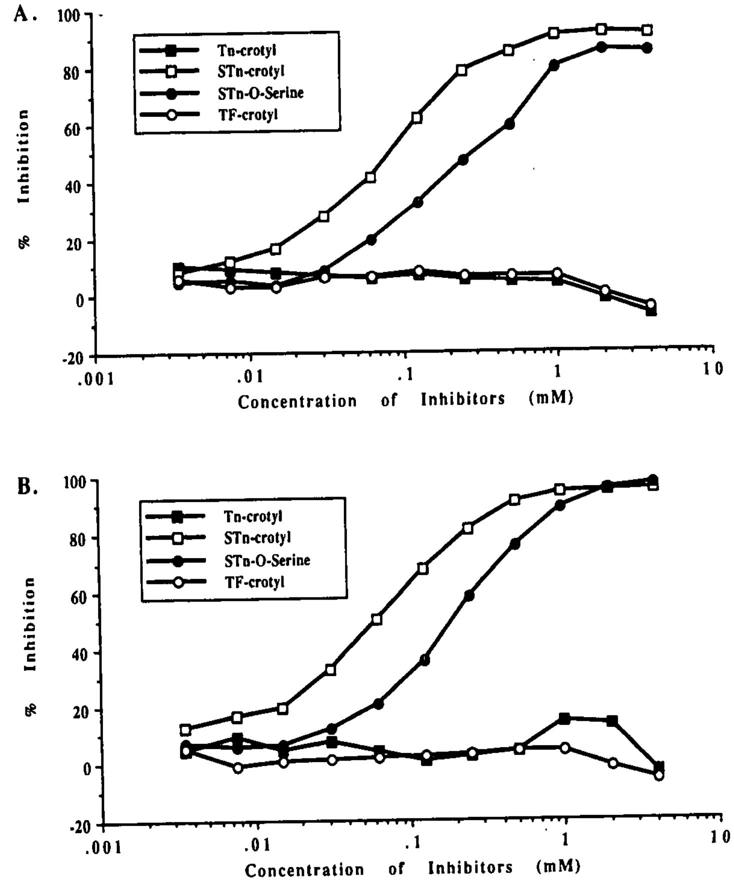


FIGURE 4. Specificities of murine IgG anti-STn antibodies were tested via a hapten inhibition method. Immune serums from two mice were preincubated for 1 hour with haptens, including STn-crotyl, STn-O-serine, Tn crotyl, and TF crotyl, before transfer onto an STn-crotyl HSA-coated solid phase (**A**) or on an ovine submaxillary mucin solid phase (**B**). Data are expressed as % inhibition relative to phosphate-buffered saline controls. Animals were immunized 2× with 0.25 μg of STn-KLH on days 0 and 14. The immune serums tested here were obtained on day 26.

TABLE 1. Hapten Inhibition of Anti-STn MAbs Generated in Mice Immunized with STn-KLH Plus DETOX Adjuvant

	MAb B195.3R1[a]		MAb B195.7R2[a]	
Hapten[b]	STn-HSA Solid Phase	OSM Solid Phase	STn-HSA Solid Phase	OSM Solid Phase
STn crotyl	89[c]	88	97	90
STn serine	92	92	100	98
Tn crotyl	0	0	2	1
Lactose	0	0	2	0

[a] Used at a 1:10 dilution of culture supernatant.
[b] Used at 0.5 mM concentration.
[c] Percentage inhibition of binding.

hapten, but not by the related TF hapten (TABLE 4). We recently began phase II studies using the same dose of another batch of STn-KLH in patients with breast cancer. The antibody response of the second patient entered in that study demonstrated an anti-OSM IgG titer of 1:1280 following the third immunization with STn-KLH plus DETOX adjuvant. FIGURE 8 demonstrates the hapten inhibition of binding of human IgG antibodies to OSM solid phase. STn-serine hapten and STn-crotyl hapten show comparable inhibition of binding (in the micromolar range of hapten) of the serum anti-STn IgG. TF-crotyl hapten did not inhibit binding, but some cross-reaction with Tn-crotyl hapten was noted (FIG. 8). The specificity of the IgG antibodies for the STn determinant was further confirmed by the

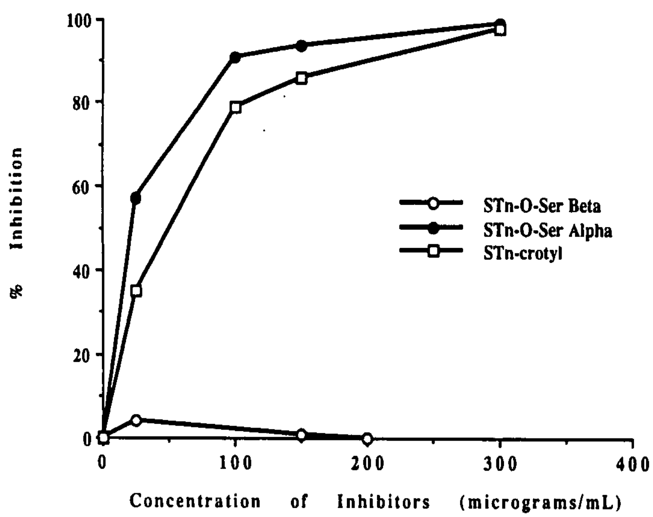


FIGURE 5. Hapten specificity of MAb B195.3R1 was tested by hapten inhibition on an ovine submaxillary mucin solid phase. The STn-crotyl hapten is compared to STn-O-serine alpha linkage (e.g., natural) and to STn-O-serine beta linkage.

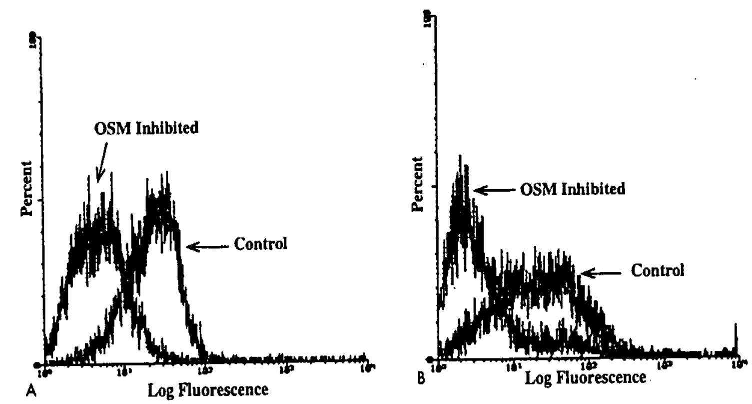


FIGURE 6. Cytofluorographic demonstration of anti-STn MAbs binding to Daudi cells. The reference MAb B72.3 is shown in **A** along with the results obtained when ovine submaxillary mucin (OSM) is included in the staining reaction, thereby inhibiting the binding. In **B**, the new anti-STn MAb B195.3 is similarly shown. Note the complete inhibition of binding observed for both antibodies when OSM inhibitor is included in the staining reaction.

observation that neuraminidase treatment of OSM and STn-HSA eliminated the binding of the antibodies (data not shown).

Clinical Response of Patients with Breast Cancer after Immunization with STn-KLH Plus DETOX Adjuvant

Evaluation of clinical efficacy in a small pilot study of only 12 patients is difficult. Five patients are alive 12 or more months and another four patients are alive 6 or more months after entry into the study. All three patients with known widespread bulky disease progressed despite ASI, two having died from widespread cancer. Two patients had partial responses, each lasting 6 months. Although several patients had disease stability for 3–13.5 months, one patient with pulmonary metastases remains stable 18 months after entry into the program (TABLE 5).

TABLE 2. Ovine Submaxillary Mucin (OSM) Inhibition of Binding of Anti-STn MAb B195.2H5 to Tumor Cells that Express STn: Comparison to Reference MAb B72.3

		OSM Concentration (μg/mL)[a]			
MAb	FCS	0.1	1	5	10
B72.3	88.8[b]	93.7	22.0	20.4	19.1
B195.2H5	81.3	68.3	52.8	40.8	29.2

[a] MAbs were incubated with various concentrations of OSM, or FCS as a negative control inhibitor, before binding of the MAbs to Daudi tumor cells.
[b] % Daudi tumor cells bound by FACS analysis.

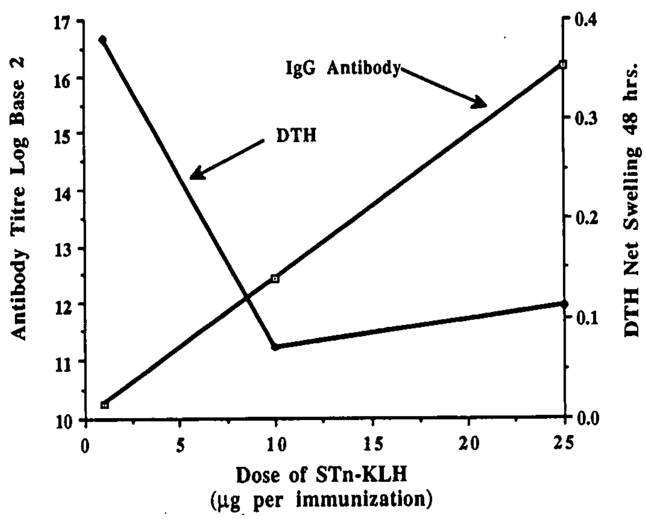


FIGURE 7. Results of delayed type hypersensitivity (DTH) and antibody responses in groups of mice immunized 3 times with 1, 10, and 25 μg of STn-KLH are compared. Five mice in each group were immunized 3 times with the various doses on days 0, 14, and 28. DTH response is expressed as net swelling of test footpad where control footpad swelling is subtracted. Test immunogen is STn-crotyl conjugated to HSA. Antibody response is expressed as the log base 2 titration result using 2 times the preimmune value as the cut-off for a positive result. DTH result for the 1-μg group is significantly different ($p < 0.05$) from that of the 25-μg group. Antibody results for the 1-μg group are significantly lower than those for the 25-μg group ($p < 0.05$). The 10-μg group antibody response is significantly lower than that of the 25-μg group ($p < 0.05$).

TABLE 3. Maximum Antibody Titers Obtained in Breast Cancer Patients Immunized with STn-KLH Plus DETOX

Patient Number	Number of Immunizations	STn-HSA Test Antigen IgM	STn-HSA Test Antigen IgG	OSM Test Antigen IgM	OSM Test Antigen IgG
1	5	2,500	1,280	1,280	320
2	4	2,560	1,280	640	80
3	4	640	320	1,280	160
4	8	2,560	2,560	10,240	80
5	7	640	1,280	1,280	640
6	2	5,120	20,480	2,560	80

Titers are with HSA background OD substracted. Titer is expressed as reciprocal of dilution of which OD exceeded 0.12 OD units.

ABBREVIATIONS: HSA = human serum albumin; OD = optical density; OSM = ovine submaxillary mucin.

TABLE 4. Specificity of Complement-Mediated Cytotoxicity Induced by TF-KLH *vs* STn-KLH Immunization

Serum from Patient Immunized with:	Hapten Inhibitor PBS	Hapten Inhibitor TF	Hapten Inhibitor STn
TF-KLH plus DETOX	42 ± 7[a]	4 ± 2	40 ± 4
STn-KLH plus DETOX	29 ± 2[b]	32 ± 2	3 ± 3

[a] % ^{51}Cr release from TF-bearing cancer cells.
[b] % ^{51}Cr release from STn-bearing cancer cells.
ABBREVIATIONS: PBS = phosphate-buffered saline solution; TF = Thomsen-Friedenreich.

DISCUSSION

The use of synthetic antigens as immunogens for active specific immunotherapy raises the question of the relevance of the synthetic antigen as a proper mimic of the natural epitope. With the sialyl-Tn epitope, both the natural and the synthetic disaccharide NANAα(2→6)GalNAcα-O are exactly the same based on ^{13}C and ^{1}H nuclear magnetic resonance spectral studies of both natural disaccharide cleaved from OSM,[15,16] the disaccharide serine structures synthesized by Lijima and Ogawa[19] and by us, and our disaccharide with the crotyl linker arm.[12] In addition, both well-characterized anti-STn MAbs B72.3[1] and TKH2[6] react specifically with our synthetic STn-crotyl epitope. Furthermore, the anti-STn MAb

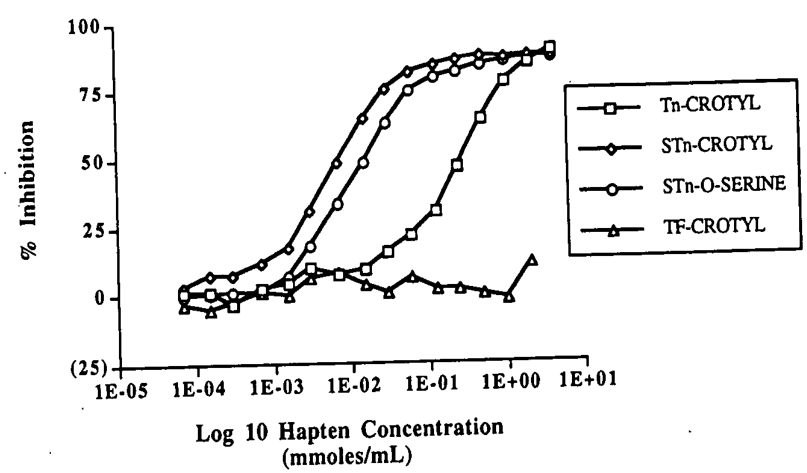


FIGURE 8. Human anti-STn IgG response is examined for hapten specificity via a hapten inhibition assay. The IgG sample is preincubated for 1 hour with the indicated concentrations of STn-crotyl, Tn crotyl, STn-O-serine, and TF-crotyl haptens, before transfer onto ovine submaxillary mucin solid phases. Data are presented as percentage inhibition relative to phosphate-buffered saline solution control.

described here binds to tumor cells in an equivalent manner to these well-characterized anti-STn MAbs and show OSM specific inhibition of that binding.

We employed a two-carbon crotyl linker arm in our synthetic hapten STn-crotyl for conjugation to KLH to generate our STn-KLH ASI vaccine. The crotyl linker is slightly different from the natural "linker" which attaches the disaccharide to mucins through an O-linked serine (FIG. 3). In the present study, we attempted to analyze whether the specificities of the IgG antibodies generated using the STn-crotyl structure recognized the "unnatural" crotyl linker arm versus the "natural" O-linked serine on mucins. Thus, we examined whether significant differences in antibody were elicited after immunization with STn-crotyl-KLH in its binding to the two structures, STn-crotyl and STn-serine. In the mouse studies, high titers ($1:10^5$–$1:10^6$) of IgG binding to the natural O-linked STn determinants on OSM were generated after the third immunization with even the smallest dose of STn-KLH vaccine employed (0.25 μg per injection). Only slightly higher IgG titers were noted when tested on STn-crotyl-HSA. IgG monoclonal antibodies generated from these mice showed excellent binding to OSM as well as to tumor cells expressing STn. Both STn crotyl and STn-serine haptens produced essentially equivalent complete inhibitions of binding of these MAbs to OSM, demonstrating the specificity of the MAbs against the disaccharide sugars of STn and demonstrating that the crotyl "linker arms" contributed less than one-half log to the binding of the antibodies. Furthermore, the mouse IgG polyclonal antibodies generated using the STn-crotyl-KLH immunogen which bind to STn-crotyl-HSA were also equally inhibited by STn-crotyl and STn-serine haptens. Thus, evidence strongly suggests that the murine IgG response to STn-crotyl-KLH involves little recognition of the crotyl linker arm and results in the generation of high titers of IgG antibodies recognizing the natural STn determinant on mucin molecules.

TABLE 5. Summary of Clinical Outcome in Breast Cancer Patients Immunized with STn-KLH Plus DETOX Adjuvant

Response Status	No. of Patients	Time to Progression (mo)
Partial response	2	6, 7
Stable	5[a]	4.5, 7, 10.5
Mixed response	2	2, 4
Progressive disease (PD)	3[b]	PD

[a] Two patients still stable at 13.5 and 18 months.
[b] These patients entered the study with bulky disease.

In patients with breast cancer immunized with the STn-KLH vaccine, the anti-STn titers generated were lower than those in mice immunized with the same vaccine. For example, the median IgG titer tested on an STn-HSA solid phase was 1:1280 and on an OSM solid phase[16] was 1:80. The higher titers observed in the mice could reflect an altered state of immune regulation due to the use of inbred mice, or the lower titers in patients could also be the result of an altered state of immune regulation due to the presence of breast cancer. Alternatively, this could reflect the *in vivo* absorption of the antibodies by STn structures on the tumor mass or secreted mucin molecules. This is being investigated. Similar to the mouse model, the human antibodies show little or no contribution by the crotyl linker arm to antibody affinity or specificity.

Itzkowitz and coworkers[5] were the first to suggest that expression of the STn epitope on colon cancer is associated with a poor prognosis. Recently, a paper by Kobayashi and coworkers[7] demonstrated that the presence of the STn epitope on circulating mucins was a strong and independent predictor of poor prognosis in patients with ovarian cancer independent of tumor grade, stage, or histologic subtype. These workers found that STn serum-negative ovarian cancer patients had a 5-year survival of approximately 85% *vs* approximately 10% for STn-positive patients. Such a strong association of STn with prognosis suggests that the STn epitope may have functional significance in the metastatic cascade.[5,7,20]

Evidence from many animal models suggests that cell-mediated immunity is the most important immune response associated with tumor regression seen after biomodulation. Indeed, when considering mucin peptide epitopes as targets for immunotherapy, evidence supports the importance of cell-mediated immunity.[21] It is reasonable to suggest that cell-mediated immunity directed against cell surface mucin peptide epitopes on carcinoma cells might be expected to attack already established metastases and reduce the size of or eliminate these lesions.[21,22] We have provided evidence that antipeptide DTH reactions may lead to "bystander killing" of even antigen-negative tumor cell variants.[21]

However, antibodies induced against carbohydrate epitopes like STn may inhibit tumor cell invasion or metastasis[5,20] and may also possibly trigger antibody-mediated cytotoxicity and inflammatory reactions. Of relevance, it was shown that specific anti-SLe^a and anti-SLe^x antibodies can inhibit tumor cells from binding to the E-Selectin on activated endothelial cells,[13] whereby it would presumably inhibit metastasis by blocking this key step of transendothelial migration.

From our small pilot studies to date, it could be misleading or erroneous to reach conclusions about any clinical efficacy of the STn-KLH plus DETOX formulation for active specific immunotherapy of breast cancers. However, the two partial responses and the apparent prolonged disease stability in some of the other patients are encouraging. The two mixed responses challenge us to continue to develop multiepitopic formulations to induce polyvalent responses against heterogeneous cancers.

One point that is still not clear is whether cyclophosphamide pretreatment enhances the immune response to our active specific immunotherapy formulation in humans. Previous results from our murine studies suggested that cyclophosphamide was important to inhibit or abrogate the T-suppressor activity believed induced by cancer-secreted mucins.[18] Current studies are investigating different strategies of utilizing cyclophosphamide as well as comparisons with and without cyclophosphamide. There may also be better methods to modulate the T-suppressor cell activity induced by cancer-associated mucins.

With the use of synthetic mimics of cancer mucin epitopes in active specific immunotherapy in both mice and humans with cancers to study specific IgG responses may lead to a clearer understanding of the role of mucins in the biomodulation of cancers. It is our intention to develop multiepitopic formulations targeting carbohydrate, peptide, and glycopeptide mucin epitopes that we believe could be clinically effective for the control of common antigenically heterogeneous human cancers.

SUMMARY

We generated a synthetic epitope, NANAα(2–6)GalNAcα-O-Crotyl (STn-crotyl), designed to "mimic" the natural O-linked epitope expressed on human

carcinoma cells, NANAα(2–6)GalNAcα-O-Serine (STn-serine). STn-crotyl was conjugated to the carrier protein KLH through the crotyl linker arm, and a "vaccine" containing STn-KLH plus DETOX adjuvant was formulated. The immunogenicity of the vaccine was evaluated preclinically in CAF_1 mice and subsequently in patients with metastatic breast cancer. The specificity and titers of IgG antibodies were evaluated by kinetic ELISA on synthetic STn-HSA and on ovine submaxillary mucin (OSM) solid phases. Ovine submaxillary mucin is a convenient source of repeating, natural O-linked STn-serine structures. Mice immunized three times with as little as 0.25 μg of STn-KLH produced IgG titers ranging from 1 : 10^4 to 1 : 10^5 when tested on solid phase OSM. Anti-OSM IgG, both polyclonal and monoclonal antibodies, generated from these mice were completely inhibited in their binding to solid phase OSM equally well by STn-serine and STn-crotyl synthetic haptens but not by several other closely related synthetic haptens. These monoclonal antibodies also bound to STn determinants on human tumor cell surfaces. Breast cancer patients immunized with 100 μg of the same vaccine produced median peak IgG titers 1 : 1280 measured on STn-HSA and 1 : 160 on OSM. Hapten inhibition experiments with the human sera demonstrated the specificities of the IgG antibodies for STn-crotyl and STn-serine, but not against several other related synthetic haptens. We found little evidence that the artificial linker arm (crotyl linker) contributed substantially to either the titer or affinity of the antibodies generated in either mice or human breast cancer patients. This suggests that the antibodies recognized the cancer-associated disaccharide NANAα(2→6)-GalNAc. Small but not large doses of STn-KLH immunogen induced anti-STn DTH responses in mice that were inversely proportional to the antibody responses. Evidence of a clinical response was noted in some of the immunized breast cancer patients, with other patients showing prolonged disease stability.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Bobina Widtman, Laurie Baronic, Marnie Wilson, and Brenda Christian for expert technical assistance.

REFERENCES

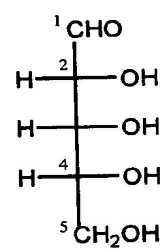
1. Hanisch, F. G., G. Uhlenbruek, H. Egge & J. Peter-Katalinic. 1989. A B72.3 second-generation monoclonal antibody (CC49) defines the mucin-carried carbohydrate epitope Galβ(1→3)[NeuAcα(2→6)]. J. Biol. Chem. **370:** 21.
2. Springer, G. F. 1984. T and Tn, general carcinoma autoantigens. Science **224:** 1198.
3. Zhuang, D., S. Yousefi & J. W. Dennis. 1991. Tn antigen and UDP-Gal : GalNAcα-R β1-3galactosyltransferase expression in human breast carcinoma. Cancer Biochem. Biophys. **12:** 185.
4. Itzkowitz, S. H., M. Yuan, C. K. Montgomery, T. Kjeldsen, H. K. Takahashi, W. L. Bigbee & Y. S. Kim. 1989. Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer. Cancer Res. **49:** 197.
5. Itzkowitz, S. H., E. J. Bloom, W. A. Kokal, G. Modin, S.-I. Hakomori & Y. S. Kim. 1990. Sialosyl-Tn: A novel mucin antigen associated with prognosis in colorectal cancer patients. Cancer **66:** 1960.
6. Kjeldsen, T., H. Clausen, S. Hirohashi, T. Ogawa, H. Iijima & S. Hakomori. 1988. Preparation and characterization of monoclonal antibodies directed to the tumor-associated O-linked sialosyl 2-6-α-N-acetylgalactosaminyl (sialosyl-Tn) epitope. Cancer Res. **48:** 2214.

7. Kobayashi, H., T. Toshihiko & Y. Kawashima. 1992. Serum sialyl Tn as an independent predictor of poor prognosis in patients with epithelial ovarian cancer. Clin. Oncol. **10:** 95.
8. Leathem, A. J. & S. A. Brooks. 1987. Predictive value of lectin binding on breast cancer recurrence and survival. Lancet **i:** 1054.
9. Price, M. R., A. J. Clarke, J. F. R. Robertson, C. O'Sullivan, R. W. Baldwin & R. W. Blamey. 1990. Detection of polymorphic epithelial mucins in the serum of systemic breast cancer patients using the monoclonal antibody, NCRC-11. Cancer Immunol. Immunother. **31:** 269.
10. Fung, P. Y. S., M. Madej, R. Koganty & B. M. Longenecker. 1990. Active specific immunotherapy of a murine mammary adenocarcinoma using a synthetic tumor-associated glycoconjugate. Cancer Res. **50:** 4308.
11. MacLean, G. D., M. B. Bowen-Yacyshyn, J. Samuel, A. Meikle, G. Stuart, J. Nation, S. Poppema, M. Jerry, R. Koganty, T. Wong & B. M. Longenecker. 1992. Active immunization of human ovarian cancer patients against a common carcinoma (Thomsen-Friedenreich) determinant using a synthetic carbohydrate antigen. J. Immunother. **11:** 292.
12. MacLean, G. D., M. Reddish, R. R. Koganty, T. Wong, S. Gandhi, M. Smolenski, J. Samuel, J. M. Nabholtz & B. M. Longenecker. 1993. Immunization of breast cancer patients using a synthetic sialyl-Tn glycoconjugate plus DETOX™ adjuvant. Cancer Immunol. Immunother. **36:** 215.
13. Magnani, J. L. 1991. The tumor markers, Sialyl Le^a and Sialyl Le^x bind ELAM-1. Glycobiology **1:** 318.
14. Mitchell, M. S., J. Kan-Mitchell, R. A. Kempf, W. Harle, H. Shau & S. Lind. 1988. Active specific immunotherapy for melanoma: Phase I trial of allogeneic lysates and a novel adjuvant. Cancer Res. **48:** 5883.
15. Gerken, T. A. 1986. The solution structure of mucin glycoproteins: Proton NMR studies of native and modified submaxillary mucin. Arch. Biochem. Biophys. **247:** 239.
16. Tsuji, T. & T. Ogawa. 1986. Carbohydrate structures of bovine submaxillary mucin. Carbohydrate Res. **151:** 391.
17. Langlois, A. J., W. D. Holder, Jr., J. D. Iglehart, W. A. Nelson-Rees, S. A. Wells, Jr. & D. P. Bolognesi. 1979. Morphological and biochemical properties of a new human breast cell line. Cancer Res. **39:** 2604.
18. Fung, P. Y. S. & B. M. Longenecker. 1991. Specific immunosuppressive activity of epiglycanin, a mucin-like glycoprotein secreted by a murine mammary adenocarcinoma (TA3-Ha). Cancer Res. **51:** 1170.
19. Iijima, H. & T. Ogawa. 1988. Total synthesis of 3-0-[-2-acetamide-6-0-(*N*-acetyl-α-D-neuraminyl-2-deoxy-α-D-galactopyronosyl]-L-serine and a stereo isomer. Carbohydrate Res. **172:** 183.
20. Ogawa, H., M. Inoue, O. Tanizawa, M. Miyamoto & M. Sakurai. 1992. Altered expression of sialyl-Tn, Lewis antigens and carcinoembryonic antigen between primary and metastatic lesions of uterine cervical cancers. Histochemistry **97:** 311.
21. Ding, L., E.-N. Lalani, M. Reddish, R. Koganty, T. Wong, J. Samuel, M. B. Yacyshyn, A. Meikle, P. Y. S. Fung, J. Taylor-Papadimitriou & B. M. Longenecker. 1992. Immunogenicity of synthetic peptides related to the core peptide sequence encoded by the human MUC1 gene: Effect of immunization on the growth of murine mammary adenocarcinoma cells transfected with the human MUC1 gene. Cancer Immunol. Immunother. **36:** 9.
22. Hareuveni, M., C. Gautier, M.-P. Kieny, D. Wreschner, P. Chambon & R. Lathe. 1990. Vaccination against tumor cells expressing breast cancer epithelial tumor antigen. Proc. Natl. Acad. Sci. USA **87:** 9498.

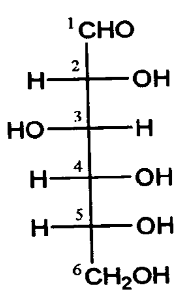
CLASS 536, ORGANIC COMPOUNDS -- PART OF THE CLASS 532-570 SERIES

SUBCLASSES

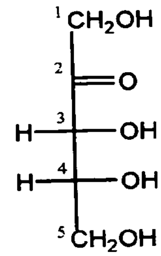
1.11 Carbohydrates and derivatives:
This subclass is indented under subclass 1. Compounds which are carbohydrates or derivatives thereof, i.e., those compounds which satisfy one of the three criteria set forth below (unless otherwise indicated, figures are representative examples only):
Criterion 1: compounds whose monomeric units are polyhydroxy mono-aldehydes [1, 2] or polyhydroxy mono-ketones [3, 4] having the formula $C_n(H_2O)_n$ (wherein n = 5 or 6 – subsequent references to "n" refer to these values), i.e., *"acyclic saccharides,"* of which representative samples are:



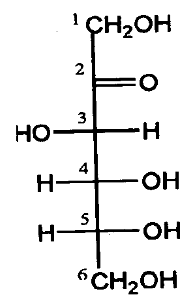
D-ribose [1]



D-glucose [2]

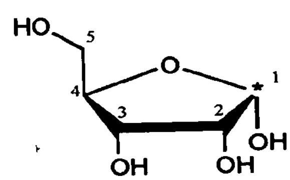


D-ribulose [3]

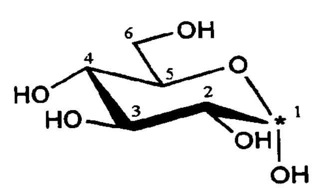


D-fructose [4]

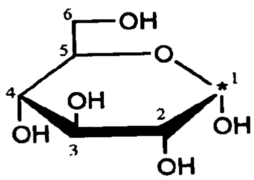
Criterion 2: the corresponding cyclic hemiacetals, i.e. *"cyclic saccharides"* of which representative examples are:



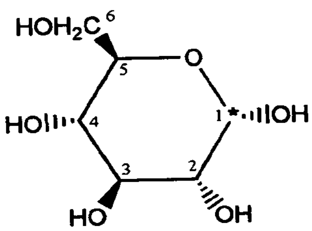
α-D-ribose D-ribofuranose [5]



α-D-glucose D-glucopyranose [6]



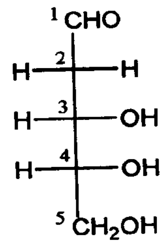
α-D-glucose D-glucopyranose [7]



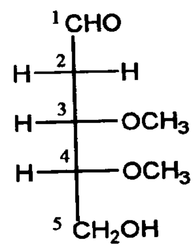
α-D-glucose D-glucopyranose [8]

wherein:
(i) figure [5] is the cyclic form of figure [1], (ii) figures [6-8] each depict, in a different way, α-D-glucose, which is one of the two cyclic forms of D-glucose shown in figure [2], (iii) figure [2] is a Fischer projection, figure [6] is a conformational projection, figure [7] is a Haworth projection, and figure [8] is a Mills projection, (iv) the number one carbon atom, the asterisked carbon atom, is the hemiacetal carbon and is also known as the anomeric carbon.

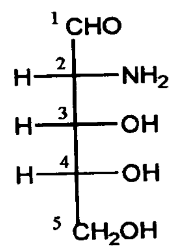
Criterion 3: the derivatives of (1) or (2) wherein:
(a) for acyclic saccharides, (i) the 5 or 6 carbon member skeleton and **the carbonyl function are not destroyed**, (ii) there are no fewer than (n - 2) total –OR moieties directly bonded to the carbon skeleton (wherein R is H or a group bonded to oxygen through carbon), (iii) no more than one oxygen atom is attached to any one carbon of the carbon skeleton, and structures [9-14] are illustrative:



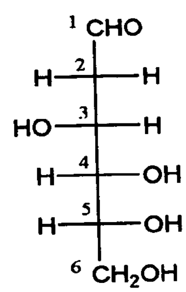
[9]



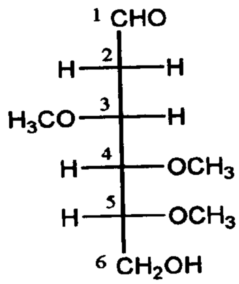
[10]



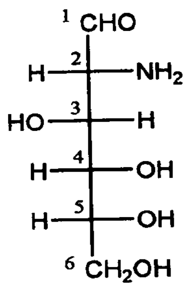
[11]



[12]



[13]

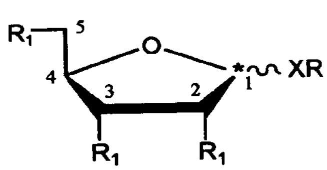


[14]

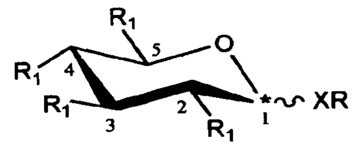
(b) for *cyclic saccharides*, except for C-glycosyl compounds described in section, and

(c) there can be substitution by moieties which do not destroy the cyclic saccharide structure as long as at least one –XH or –XR group is bonded directly to the hemiacetal/anomeric carbon (this carbon is denoted with an asterisk in the figures below), wherein X is –O–, –S–, or $-NR_S-$ and R is H or a group bonded to X through carbon and R_S is a substituent which completes the valency of the nitrogen atom,

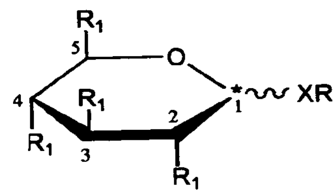
and figures [15-18] represent the minimum structure necessary to constitute a cyclic saccharide derivative:



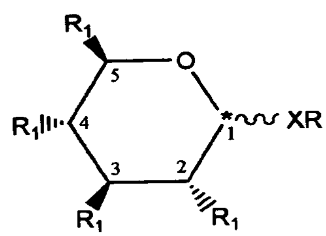
[15] n=5



[16] n=6



[17] n=6



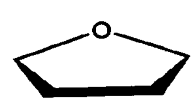
[18] n=6

wherein:

- at least one of the R_1 (where n = 5) or at least two of the R_1 (where n = 6) must be –OR wherein R is H or a group bonded to the oxygen of –OR through carbon; and the representation of the bond between XR and the ring position 1 in ech of the structures [15-18] signifies an α (i.e., axial) or β (i.e., equatorial) configuration

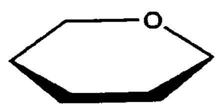
(c) for cyclic saccharide, C-glycosyl-type compounds (also known as C-glycosides)

(i) C-glycosyl compounds will have the structure defined below [19-21]



[19] n=5

[20] n=6



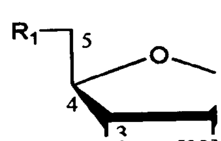
[21] n=6

wherein:
- at least one of the R_1 (where n = 5) or at least two of the R_1 (where n = 6) must be –OR wherein R is H or group bonded to the oxygen of –OR through carbon;
- R_2, R_3, and R_4 alone or in combination can be any substituent which completes the valency of the carbon atom.

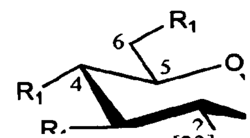
(ii) "C-glycoside", although often used in the art to represent this type compound, is a misnomer–"C-glycosyl" is the proper term.

(1) Note. All statements in this sublass definition are intended to be inclusive of all spatial and stereochemical configurations, except if otherwise specified.

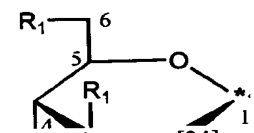
(2) Note. Oxygen heteroatom-containing cyclic compounds lacking any –XH or –XR bonded to the anomeric carbon of the cyclic structure are not carbohydrates/cyclic hemiacetals; such compounds are tetrahydrofurans [22] or tetrohydropyrans [23, 24]:



Tetrahydrofuran [22]

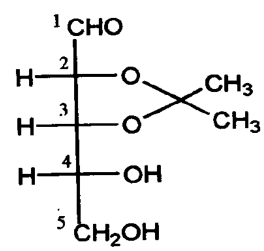


Tetrahydropyran [23]

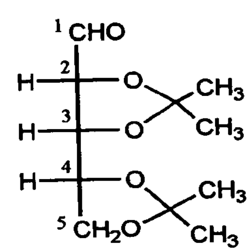


Tetrahydropyran [24]

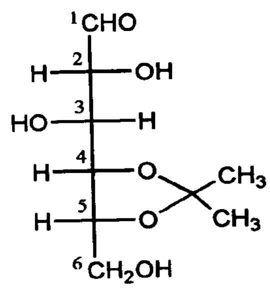
(3) Note. Included within the scope of this class are compounds wherein oxygens which are attached to carbons of the carbohydrate skeleton are also attached to the same alkylidene or substitued alkylidene groups. See, for example, structures [25-29].



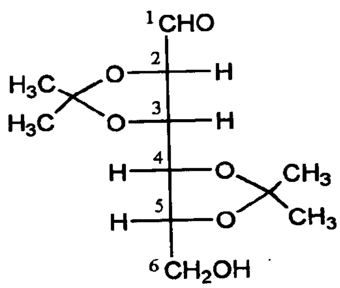
[25]



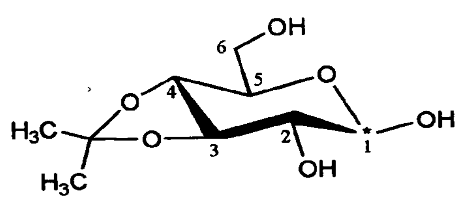
[26]



[27]



[28]



[29]

(4) Note. Carbohydrate degradation products which contain fewer than five carbon atoms in the carbohydrate moiety are not provided for in this class, but are classified elsewhere in the chemical compound area.

(5) Note. Alcohol, acid, and amine derivatives of carbohydrates which are formed by an alcohol, carboxylic acid, or amine function replacing the oxygen of the carbonyl group of an acyclic carbohydrate are not provided for in this class, but are classified elsewhere.

(6) Note. The heteroatom of the cyclic carbohydrate must be an oxygen. Compounds with different heteroatoms or compounds without a heteroatom in the cyclic structure (e.g. inositol) are not classified in this class; they are classified elsewhere.

(7) Note. Some names of common carbohydrates include:
Monosaccharides: Fructose (Fru), Fucose (Fuc), Galactosamine (GalN), Galactose (Gal), Glucosamine (GlcN), Glucose (Glc), Glucuronic acid (GlcA), Idose (Ido), Mannose (Man), Neuraminic acid (e.g., Neu5Ac, etc.), Sialic acid, Xylose (Xyl).
Oligosaccharides: Cyclodextrin, Lactose (Lac), Maltose, Raffinose, Sialyl Lewis x (sLe^x), Sucrose, Trehalose.
Homopolysaccharides (all the monomeric units are the same): Amylose/Amylopectin, Cellulose, Chitin/Chitosan, Dextran, Glucan, Inulin, Starch.
Heteropolysaccharides (different monomers comprise the repeating unit): Algin/Alginic acid, Bacterial/capsular polysaccharides; Glycosaminoglycans (Mucopolysaccharides including Chondroitin sulfate, Dermatan sulfate, Heparin, Heparan sulfate, Hyaluronic acid, Keratan sulfate); Gums, Mannans, Pectins, Xylan.

SEE OR SEARCH THIS CLASS, SUBCLASS:
22.1 through 24.5 , for nucleic acids and oligomeric or polymeric forms thereof, including DNA or RNA fragments (e.g., genes, etc.)

SEE OR SEARCH CLASS:
127, Sugar, Starch, and Carbohydrates, subclasses 36 through 41 for hydrolysis of carbohydrates by nonbiochemical methods wherein the process stops with such hydrolysis or is followed by purification, concentration, or crystallization of the sugar or sugar solution thereby produced.

as a sequence of amino acids, carbohydrate residues, or nucleic acids:
This subclass is indented under subclass 9.34. Subject matter wherein the polypeptide attached to said imaging agent directs delivery of said imaging agent to a specific in vivo target and wherein the specific binding properties of either the in vivo target or the polypeptide are described by a sequence (e.g., a series of amino acids, carbohydrate residues of a glycoprotein, nucleic acids encoding a polypeptide, etc.) or wherein the particular region of binding is described functionally (e.g., a unique region of an imaging agent defined by the fact that it binds a specific region of a target cell, but does not cross react with another region on the target cell etc.).

(1) Note. A mere recitation of a generic type of binding (e.g., "an anti-myosin antibody", etc.) would be proper for subclass 9.34, but a recitation of multiple properties of the binding site would be proper for subclass 9.341.

9.35 Carbohydrate or derivative thereof attached to or complexed with the agent:
This subclass is indented under subclass 9.3. Subject matter wherein the agent has attached or complexed to it a carbohydrate or a derivative thereof such as a monosaccharide or polysaccharide (the monosaccharide radical units of which contain at least five carbon atoms) or their reaction products wherein the carbon skeleton of the saccharide or polysaccharide is not destroyed, e.g., dextran, cellulose, glucose, etc.

9.351 The region of the imaging agent responsible for binding to an in vivo target or the region of the target responsible for binding to the agent is specifically recited functionally or as a sequence of amino acids, carbohydrate residues, or nucleic acids:
This subclass is indented under subclass 9.35. Subject matter wherein the carbohydrate attached to said imaging agent directs delivery of said imaging agent to a specific in vivo target and wherein the specific binding properties of either the in vivo target or the carbohydrate are described by a saccharide sequence or wherein the particular region of binding is described functionally (e.g., a unique region of an imaging agent defined by the fact that it binds a specific region of a target cell, but does not cross react with another region on the target cell, etc.)

9.36 Transition, actinide or lanthanide metal containing:
This subclass is indented under subclass 9.3. Subject matter wherein the agent contains a transition, actinide, or lanthanide metal (Atomic Numbers 21-30, 39-48, 57-80, and 89-106, inclusive).

(1) Note. The metals included under transition, actinide, or lanthanide metals are Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Y, Zr, Nb, Mo, Tc, Ru, Rh, Pd, Ag, Cd, La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hf, Ta, W, Re, Os, Ir, Pt, Au, Hg, Ac, Th, Pa, U, Np, Pu, Am, Cm, Bk, Cf, Es, Fm, Md, No, Lr, Unq, Unp, and Unh.

(2) Note. A complex of a metal and an organic compound is considered to be a compound per se and is classified based on the moiety (metal or organic compound) which occurs first in the classification schedule.

(3) Note. The transition, actinide or lanthanide metal may be complexed to a chelating agent.

SEE OR SEARCH THIS CLASS, SUBCLASS:
1.11+, for compositions or methods under the class definition which contain radioactive isotopes of metals e.g., U, Tc, etc., including diagnostic or test compositions or methods.

SEE OR SEARCH CLASS:
534, Organic Compounds, subclasses 15+ for nonradioactive organic lanthanide or actinide complexes, per se.

9.361 Heterocyclic compound is attached to or complexed with the metal:
This subclass is indented under subclass 9.36. Subject matter wherein the metal is attached to or complexed with a heterocyclic compound.

(1) Note. A heterocyclic compound is a compound containing at least one hetero